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PRINCIPAL INVESTIGATOR: Jeffrey W. Pollard, Ph.D.

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine  
of Yeshiva University  
Bronx, New York 10461

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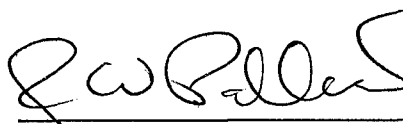
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## Introduction


Over-expression of the macrophage growth factor, colony stimulating factor-1 (CSF-1) and its receptor, the *c-fms* protooncogene product, is associated with the progression of breast cancer. The recruitment of macrophages to breast tumors is also associated with poor prognosis, and it has been suggested that these cells promote tumor growth and metastasis by secretion of angiogenic factors and through matrix remodeling activities. Since CSF-1 is the major growth factor for macrophages, we hypothesized that, in addition to possible autocrine roles in tumor cells, that CSF-1 is responsible for the recruitment of these tumor associated macrophages (TAMs). We proposed to test this hypothesis by crossing mice with a null mutation in the CSF-1 gene, osteopetrotic (*csfm<sup>op</sup>*), with transgenic mice having a predisposition to developing mammary gland tumors. Significant diminution of the growth and/or metastatic potential of these tumors would provide evidence for a causal role of CSF-1 in tumorigenesis. To confirm a role for the TAMs, we proposed to re-introduce signaling to mononuclear phagocytes in *csfm<sup>op</sup>/csfm<sup>op</sup>* mice using a gain-of-function mutation in *fms*, or to remove macrophage function in wild type mice using a dominant negative CSF-1 receptor mutation, with expression of each transgene being driven by a mononuclear-phagocytic specific promoter. These experiments, therefore, were designed to rigorously test the role of CSF-1 and TAMs in mammary gland tumorigenesis and progression.

The central hypothesis to be tested in this proposal is that CSF-1 regulated TAMs play an important role in the development and progression of mammary gland tumors. To test this, a variety of strategies were to be undertaken to cross a null mutation in the CSF-1 gene onto mammary gland tumor susceptible mice.

## Body

In task one, we proposed to examine the incidence of mouse mammary tumor virus-induced tumors in C3H mice carrying the CSF-1 null mutation (*csfm<sup>op</sup>*). This required inbreeding of the *csfm<sup>op</sup>* mutation on to a C3H background carrying the mouse mammary tumor virus (MMTV) and this was achieved to the sixth generation of inbreeding as proposed. The females *+csfm<sup>op</sup>* C3H MMTV+ have been crossed with male *csfm<sup>op</sup>/csfm<sup>op</sup>* C3H MMTV+ mice, and the *csfm<sup>op</sup>/csfm<sup>op</sup>* offspring and their littermate controls have been examined for the incidence of mammary tumor development over their life-span. So far, 12 out of 12 *+csfm<sup>op</sup>* C3H MMTV+ mice developed mammary tumor in 7 months versus 1 out of 5 *csfm<sup>op</sup>/csfm<sup>op</sup>* C3H MMTV+. However, difficulty in propagating this inbred strain is currently curtailing the possibility of generating enough old female mice to continue this study. Currently, we are trying several strategies to improve the breeding performance of these mice.

In the second task, we proposed to cross the *csfm<sup>op</sup>* allele onto mammary tumor transgenic strains. Because of the high penetrance of the MMTV-middle T transgene in causing mammary tumors, we concentrated initially on this strain. However, we have



also crossed the *csfm*<sup>op</sup> mutation onto the MMTV-neu strain (we chose this rather than the originally proposed MMTV-cyclin D1 both because of its availability but also because of its mimic of a human oncogene).

Using mammary gland whole mounts, we have analyzed the onset and the development of breast tumors in *csfm*<sup>op</sup>/*csfm*<sup>op</sup> and wild-type (+/*csfm*<sup>op</sup>) mice carrying the MMTV/PYVmT transgene. The results have shown that in both *csfm*<sup>op</sup>/*csfm*<sup>op</sup> and +/*csfm*<sup>op</sup> female mice carrying the transgene, the mammary gland tumors developed at an early age (tumors were detected in mammary glands at the age of four-weeks which is the earliest age examined) and in all of the mammary glands examined. However, the progression of the tumor in the *csfm*<sup>op</sup>/*csfm*<sup>op</sup> mammary gland appeared delayed compared to the +/*csfm*<sup>op</sup> counterpart. As shown in Fig. 1, the development of the tumor can be divided into two stages. Only focal tumors can be found around the nipple in the early stage of the development (Fig. 1A, 1B, 1D and 1F). In the late stage, multifocal tumors develop in the distal area from the nipple (for example, Fig. 1C, 1E and 1H). In +/*csfm*<sup>op</sup> mammary glands, only focal tumors (early stage) were detected in mice at 4-week of age. However, the percentage of +/*csfm*<sup>op</sup> mice carrying late stage tumors (multifocal) was rapidly increased during the next 4 weeks and all of the mammary glands examined developed multifocal tumors by 8-week of age (Fig. 1C and 1E. Fig. 2). On the other hand, when the *csfm*<sup>op</sup>/*csfm*<sup>op</sup> littermates were examined, only focal mammary tumors were detected between 4- to 9-weeks of age (Fig 1B, 1D and 1F. Fig 2). The percentage of *csfm*<sup>op</sup>/*csfm*<sup>op</sup> mice carrying multifocal mammary tumors increased in the following few weeks (age 9- to 18-weeks) but never exceeded 60% (Fig. 2). These results suggest the progression of the tumor in the mammary gland is affected when CSF-1 is depleted in the tumor microenvironment.

To further identify the primary cause(s) of the observed difference in tumor development, we are examining several factors possibly associated with macrophages function in tumor invasion and metastasis, including macrophage infiltration, angiogenesis, and the expression of cytokines in mammary glands. Preliminary result of immunohistochemical study using a macrophage lineage marker, F4/80, has shown that there was a significant macrophage infiltration in and around the tumors in +/*csfm*<sup>op</sup> mammary glands [Fig. 3, *wt*(*mT*+) ] but only few positive stained cells were found around tumors in *csfm*<sup>op</sup>/*csfm*<sup>op</sup> mammary gland [Fig. 3, (*csfm*<sup>op</sup>/*csfm*<sup>op</sup>(*mT*+) ] .

To determine the effect of CSF-1 depletion in the distal metastasis of the mammary gland tumors, lungs from +/*csfm*<sup>op</sup> and *csfm*<sup>op</sup>/*csfm*<sup>op</sup> tumor-carrying mice have been prepared and the examination of mammary gland metastases is in progress.

It became apparent that we needed greater information about the development of the *csfm*<sup>op</sup>/*csfm*<sup>op</sup> mammary gland compared to wild type. In another study, we examined WT and *csfm*<sup>op</sup>/*csfm*<sup>op</sup> mice's mammary glands from day 18 to 12 weeks of age (figure 4). The fourth inguinal mammary gland whole mounts of 3 to 5 mice were analyzed and the different parameters such as duct length, branching number, terminal end bud (TEB)

number were quantified (figure 5). At 18 days postpartum, WT and mutant mammary glands display the same rudimental branching, while the fat pad size is dramatically reduced in *csfm<sup>op</sup>/csfm<sup>op</sup>* versus wt mammary gland. TEB formation, the initial structure for duct elongation, is delayed in *csfm<sup>op</sup>/csfm<sup>op</sup>* mammary gland. The first TEB appear at 3 weeks in WT mammary glands compared to 4 weeks in the mutant *csfm<sup>op</sup>/csfm<sup>op</sup>*, and the TEB number in WT mammary gland is significantly higher than in mutant mammary gland. This is closely related to a more extensive ductal tree and a larger fat pad size in *+csfm<sup>op</sup>* mammary glands. Between 8 and 9 weeks, in *+csfm<sup>op</sup>* mice ductal branching filled the whole fat pad, TEB have disappeared and some secondary ducts resulting from the hormonal estrus cycle influence are formed. In contrast, the ductal tree is still growing in *csfm<sup>op</sup>/csfm<sup>op</sup>* mammary glands and finally, at 12 weeks of age, in the *csfm<sup>op</sup>/csfm<sup>op</sup>* mice the branching has filled the fat pad. However, no secondary ducts are found. Interestingly, the parameters describing the *csfm<sup>op</sup>/csfm<sup>op</sup>* aberrant mammary gland development such as, duct length, branching and TEB numbers, do not follow the difference of mouse weight observed from the very beginning of mouse life (3 weeks of age, figure 5), indicating that the *csfm<sup>op</sup>/csfm<sup>op</sup>* mammary gland phenotype is not due to a general metabolism defect but instead, due to a local mammary gland defect.

Since CSF-1 is the major growth factor involved in mononuclear phagocyte proliferation, differentiation and recruitment, we analyzed macrophage distribution during prepubertal mammary gland development. At 3 weeks of age, in *+csfm<sup>op</sup>* mammary gland, all the TEB are surrounded by macrophages (figure 6e), whereas in *csfm<sup>op</sup>/csfm<sup>op</sup>* mammary glands, few macrophages are scattered in the fat pad and, none are localized around the rudimentary tips of the duct that at this time have not yet formed TEB (figure 6f). These data indicate that macrophage recruitment is closely related to TEB formation. At 5 weeks of age, macrophage density surrounding the TEB is dramatically reduced in *csfm<sup>op</sup>/csfm<sup>op</sup>* versus WT mammary gland. When *+csfm<sup>op</sup>* and *csfm<sup>op</sup>/csfm<sup>op</sup>* mice have been treated daily for 5 weeks with CSF-1 from birth, macrophage density is not modified. However, macrophage morphology had changed from a mixture of fibroblastic (activated) and round (resting) morphology (figure 6a,c) to the spread activated shape (figure 6b,d) in WT as well as in *csfm<sup>op</sup>/csfm<sup>op</sup>* mammary glands. The CSF-1 treatment rescued the branching number and TEB number in *csfm<sup>op</sup>/csfm<sup>op</sup>* mice while the duct length still remained low compared to the non-treated WT mice (Figure 7). Our results suggest strongly that the "activated form" macrophages are closely associated with TEB and branching formation.

These data strongly support the hypothesis that CSF-1 regulated macrophage functions influence both ductal growth into the fat pad, its responsiveness to estrogens and probably through these mechanisms influence mammary gland tumor development. We believe these observations are very significant and will be vigorously pursued over the next year.

In other aims (task 3), a C3H mammary carcinoma cell line was obtained and injected into the inguinal mammary gland fat pad. Unfortunately, this evoked a host

versus graft reaction and rejection of the tumor. Thus, either our inbreeding is not sufficient, or there are different minor histocompatibility antigens expressed on the cell line which causes the immune rejection. Thus, given the success of the analysis of mammary gland tumors in the transgenic mice bearing the *csfm<sup>op</sup>* mutation, we will drop this task and concentrate on the above analysis.

The fourth task was to use the tetracycline-inducible binary system to express CSF-1 in a regulated fashion specifically in the mammary gland. We appear to be having success with this system and have identified several founders where CSF-1 expression is regulated in a tight fashion. Thus, we have begun to cross these double transgenic mice (tet-TA and tetop-CSF-1) onto the strain carrying the *csfm<sup>op</sup>* mutation and MMTV-Middle-T transgene. This breeding is complex and will continue through year two as stated. However, if successful and, regulated expression of CSF-1 within tumors can be achieved, then it will provide considerable insight into the mechanism of CSF-1 action on macrophages and the importance of this growth factor in various phases of tumor progression.

The final task was to express gain-of-function and loss-of-function mutants of the CSF-1R specifically in the mononuclear phagocytic lineage. As stated, we made transgenic mice with a FLAG-tagged *v-fms* (gain-of-function) cDNA driven by a TRAP-promoter. Six founder transgenic mice were obtained and crossed onto the *csfm<sup>op</sup>* background and interbred to obtain homozygous mutants. However, at best only limited rescue of the macrophage defect could be detected. Close analysis of primary bone marrow derived macrophages isolated from these transgenic mice showed expression of the transgene but mostly in an unspliced form (~95%). Thus, in some fashion, the cDNA was interfering with correct splicing of the TRAP intron included in the construct to enhance expression of the transgenic cDNA. Consequently, there was little to no protein synthesized. During these studies, another promoter was published that was reported to have better specificity for the mononuclear phagocytic lineage, the hMRP8 promoter. We have obtained this and made *c-fms* constructs and have recently injected these constructs into oocytes to create new founders. Meanwhile, we are trying to obtain a TRAP-minigene construct that may solve our problems of pre-mRNA splicing. These types of experiments are technically complex and at the cutting edge of transgenic technology. Nevertheless, we continue to believe that they provide unique approaches to specific cell type function. Thus, in year two, we will continue to explore this avenue of research vigorously.

## Conclusion

During the first year of funding, we have made considerable progress in our studies designed to test the hypothesis that tumor associated macrophages play an important role in promoting tumor progression and metastasis. Using genetic strategies, we have removed CSF-1, the major macrophage growth factor, from mice bearing mammary gland tumor susceptibility genes. This was shown to severely influence the

[REDACTED]

development of these tumors and this correlated with a relative absence of macrophages in the mammary gland and in the tumors that develop. This result provides considerable support for our basic hypothesis and provides the foundation for our studies in the second year of funding.

### Figure Legend

**Fig. 1.** Whole mount analysis of tumor progression in the fourth mammary gland of PYVmT transgenic mice. Mammary glands prepared from  $+/\text{csfm}^{op}$  mice at 5-, 6-, and 8-week of age: A, C, and E. Mammary glands prepared from  $\text{csfm}^{op}/\text{csfm}^{op}$  mice at 5-, 6-, 8-, 10, and 18-weeks of age: B, D, F, G, and H.

**Fig. 2.** Regional tumor development in the fourth mammary gland. Whole mount mammary glands from both  $+/\text{csfm}^{op}$  and  $\text{csfm}^{op}/\text{csfm}^{op}$  mice carrying PYVmT transgene were prepared at different ages. Mammary glands with tumors distant from nipple are scored as distal growth.

**Fig. 3.** Macrophage distribution in mammary glands. Slides are prepared from mice at 9-week of age and stained with rat anti-mouse F4/80 monoclonal antiserum.  $+/\text{Csfm}^{op}$  mice with or without PYVmT transgene;  $wt(mt+)$  and  $wt(mt-)$ .  $\text{Csfm}^{op}/\text{csfm}^{op}$  mice with or without PYVmT transgene:  $\text{csfm}^{op}/\text{csfm}^{op}(mt+)$  and  $\text{csfm}^{op}/\text{csfm}^{op}(mt-)$ .

**Fig. 4.** Whole mounts of  $+/\text{csfm}^{op}$  and  $\text{csfm}^{op}/\text{csfm}^{op}$  mammary glands. Whole mount preparations are shown for 3, 4, 9 and 12 weeks of age of  $+/\text{csfm}^{op}$  and  $\text{csfm}^{op}/\text{csfm}^{op}$  virgin mice. The photomicrographs were taken at the same magnification of the entire fourth inguinal mammary gland, showing the atrophic development in  $\text{csfm}^{op}/\text{csfm}^{op}$  mice. The arrow indicates the terminal end buds (TEB), NP is the nipple area and LN the lymph node.

**Fig. 5.** Ductal length, branching and terminal end bud (TEB) numbers and weight of  $+/op$  and  $\text{csfm}^{op}/\text{csfm}^{op}$  mice.

Mice were killed at 2.5 to 12 weeks, and the fourth inguinal mammary gland whole mounts were analyzed. The ductal length (mm) is measured from the nipple area to the tip of the longest duct through the lymph node. Branching number is the mean of branching number along the 3 longest ducts from the nipple area. TEB are quantified in the whole mammary gland. Points represent mean  $\pm$  SD of at least three mice per time point.

### Fig. 6

F4/80 immunostaining in the mammary gland of  $+/\text{csfm}^{op}$  (a,b,e) and  $\text{csfm}^{op}/\text{csfm}^{op}$  (c,d,f) mice. Sections of mammary gland from mice 5 weeks of age (a,b,c,d) and 3 weeks of age (e,f) were immunostained using anti-F4/80 antibody, and positive cells were detected with a peroxidase-coupled detection system (brown). Sections were lightly counterstained with hematoxylin. Arrows show the spread macrophages and arrow heads the round macrophages. b,d) mice treated with CSF-1 from birth.

### Fig. 7

Ductal length, branching and terminal end bud (TEB) numbers of  $+/\text{csfm}^{op}$  and  $\text{csfm}^{op}/\text{csfm}^{op}$  mice. Mice treated with CSF-1 from birth and their untreated littermates were killed at 5 weeks, and the fourth inguinal mammary gland whole mounts were analyzed. The ductal length (mm) is measured from the nipple area to the tip of the longest duct through the lymph node. Branching number is the mean of branching number along the 3 longest ducts from the nipple area. TEB are quantified in the whole mammary gland. Points represent mean  $\pm$  SD of at least three mice per time point.

**Fig. 1**

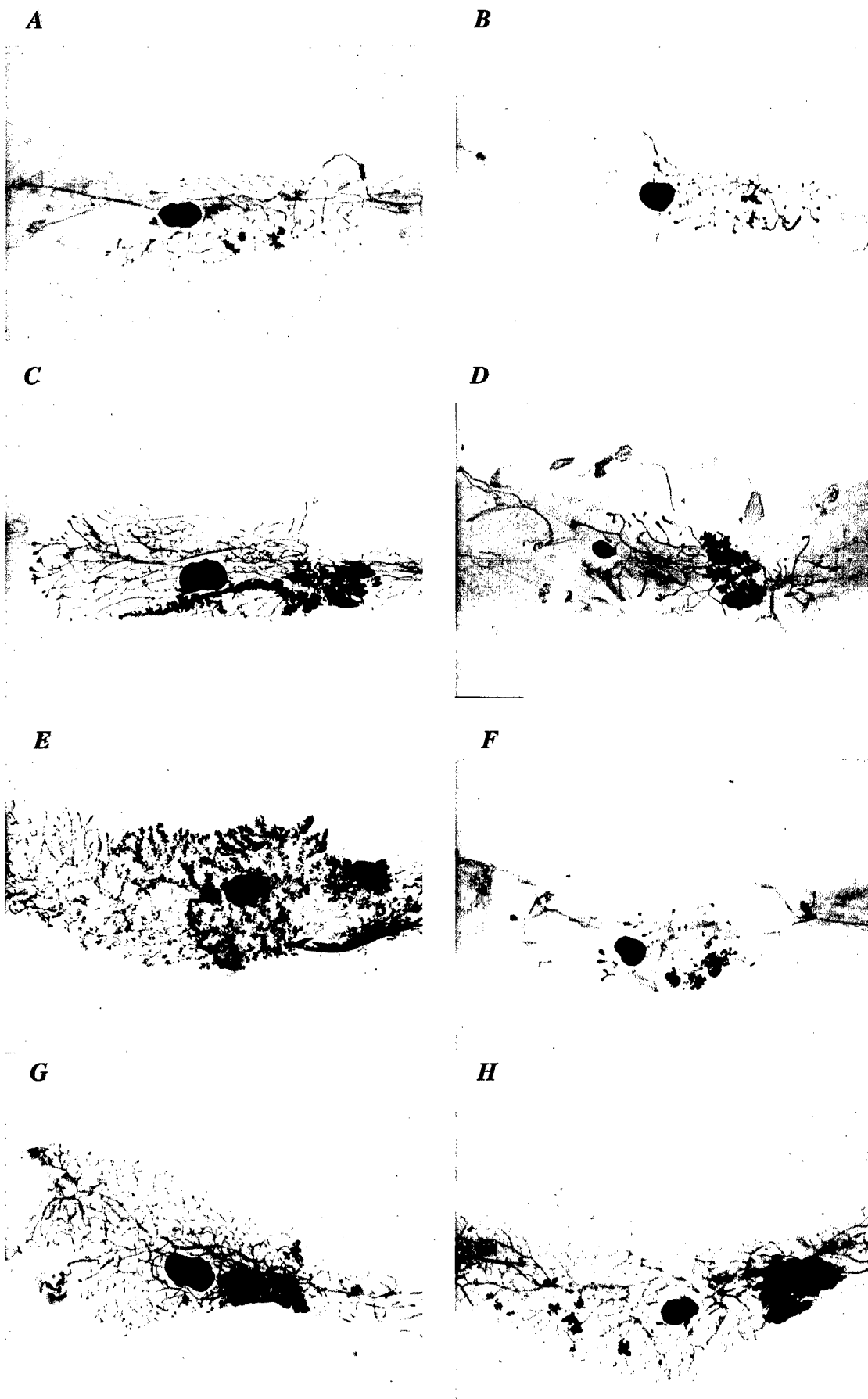


Figure 2

## Distal Tumor Growth in Mammary Gland

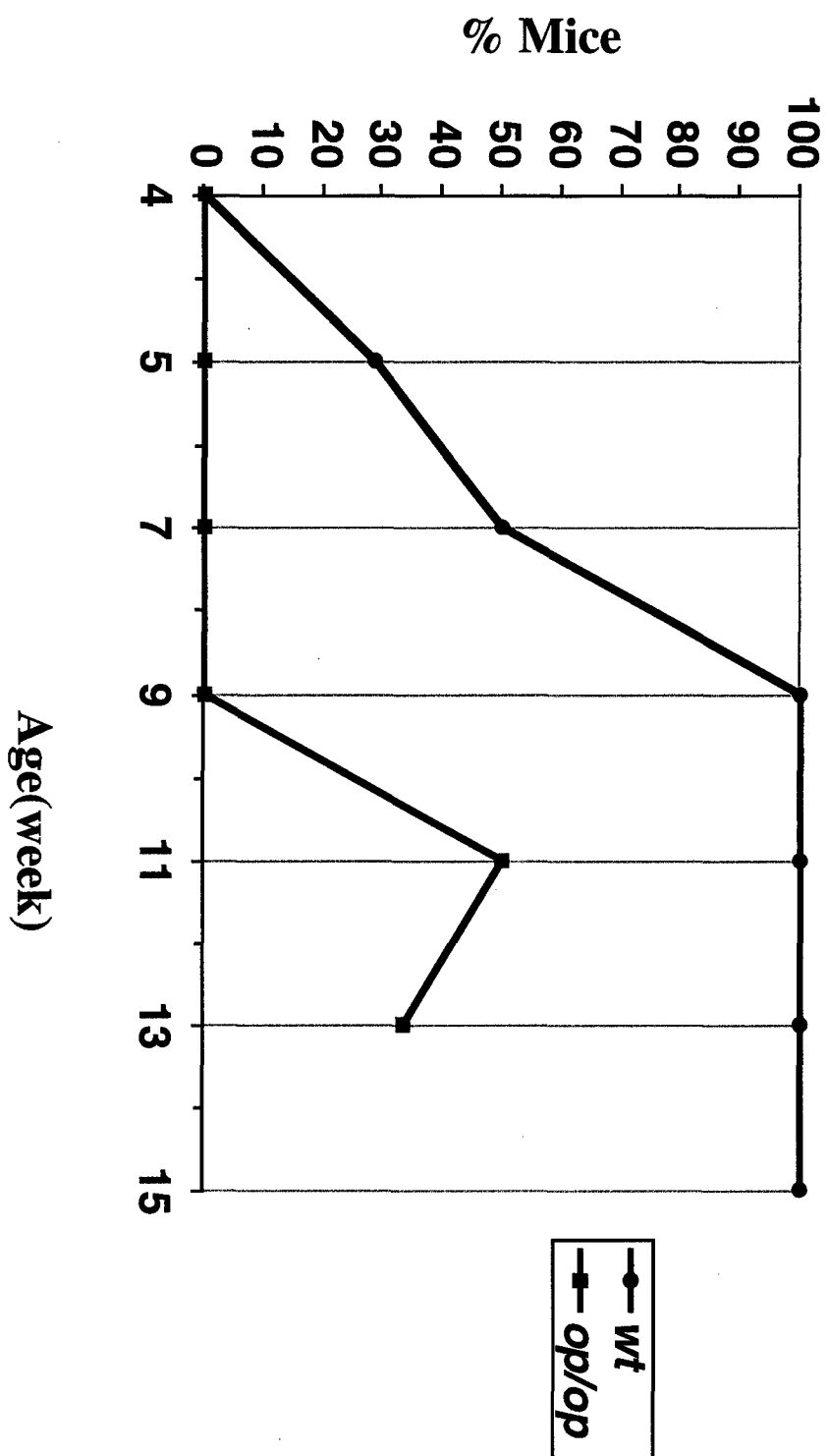
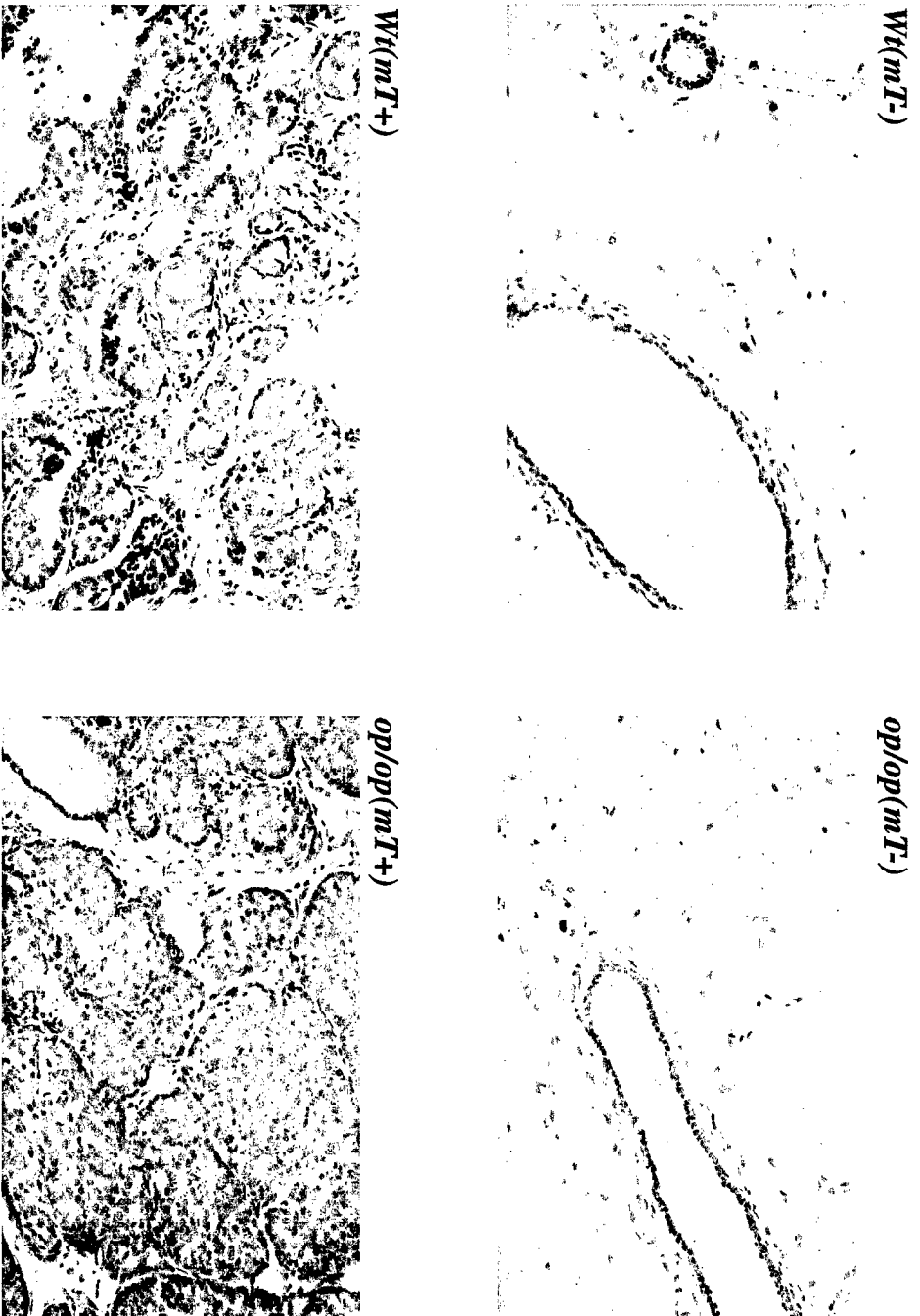
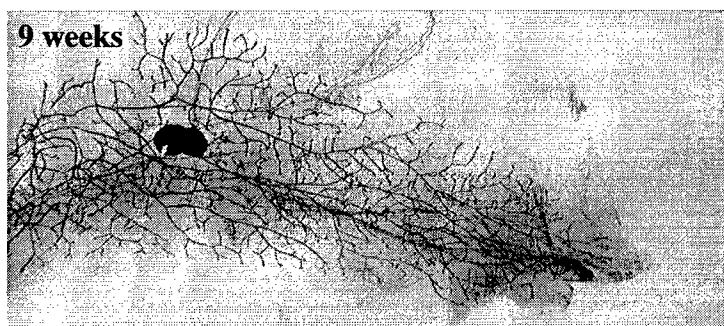
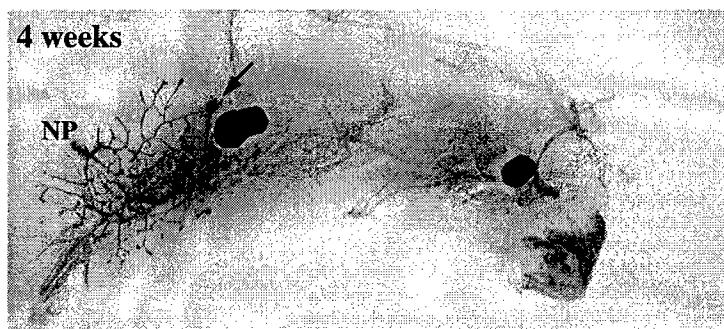
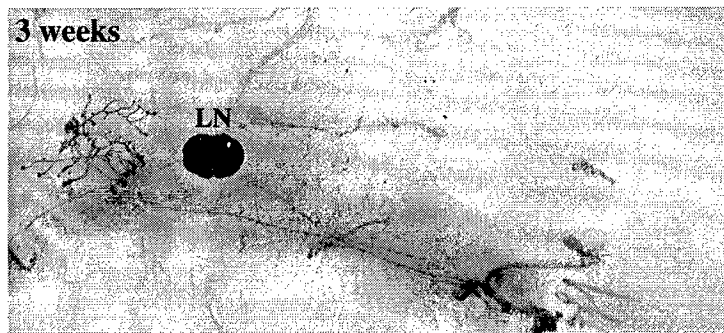


Fig. 3

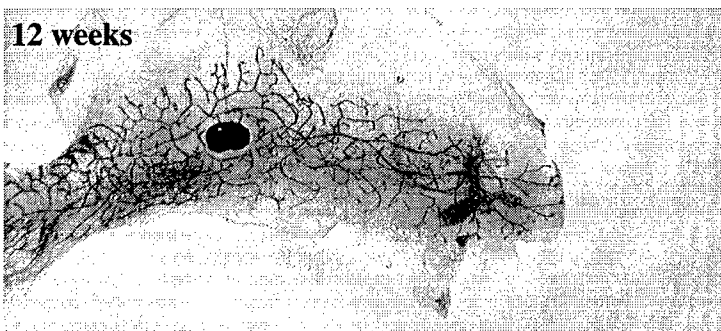
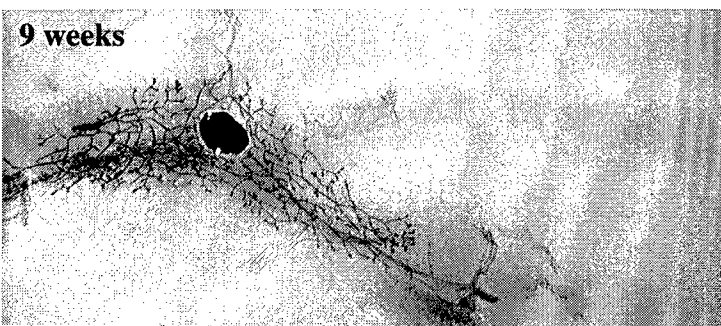
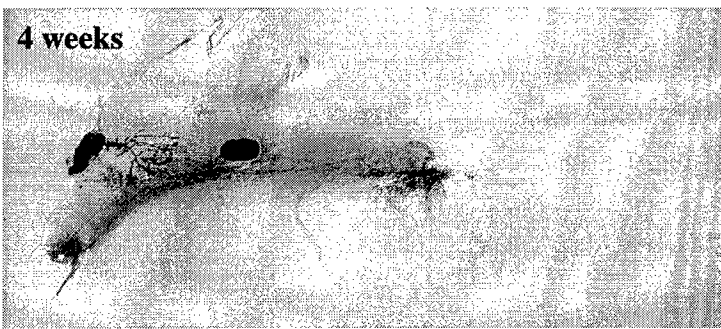
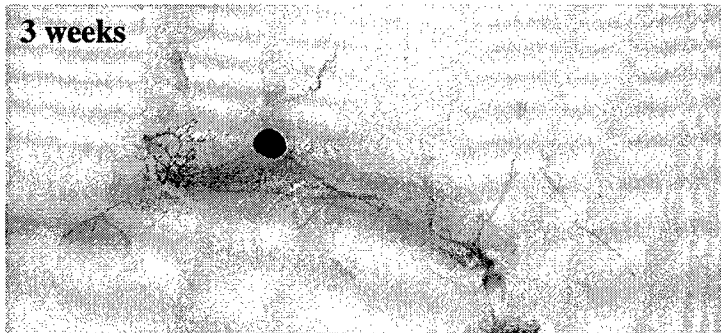


**Figure 4**

*+/csfm<sup>op</sup>*



*csfm<sup>op</sup>/csfm<sup>op</sup>*



**Figure 5**

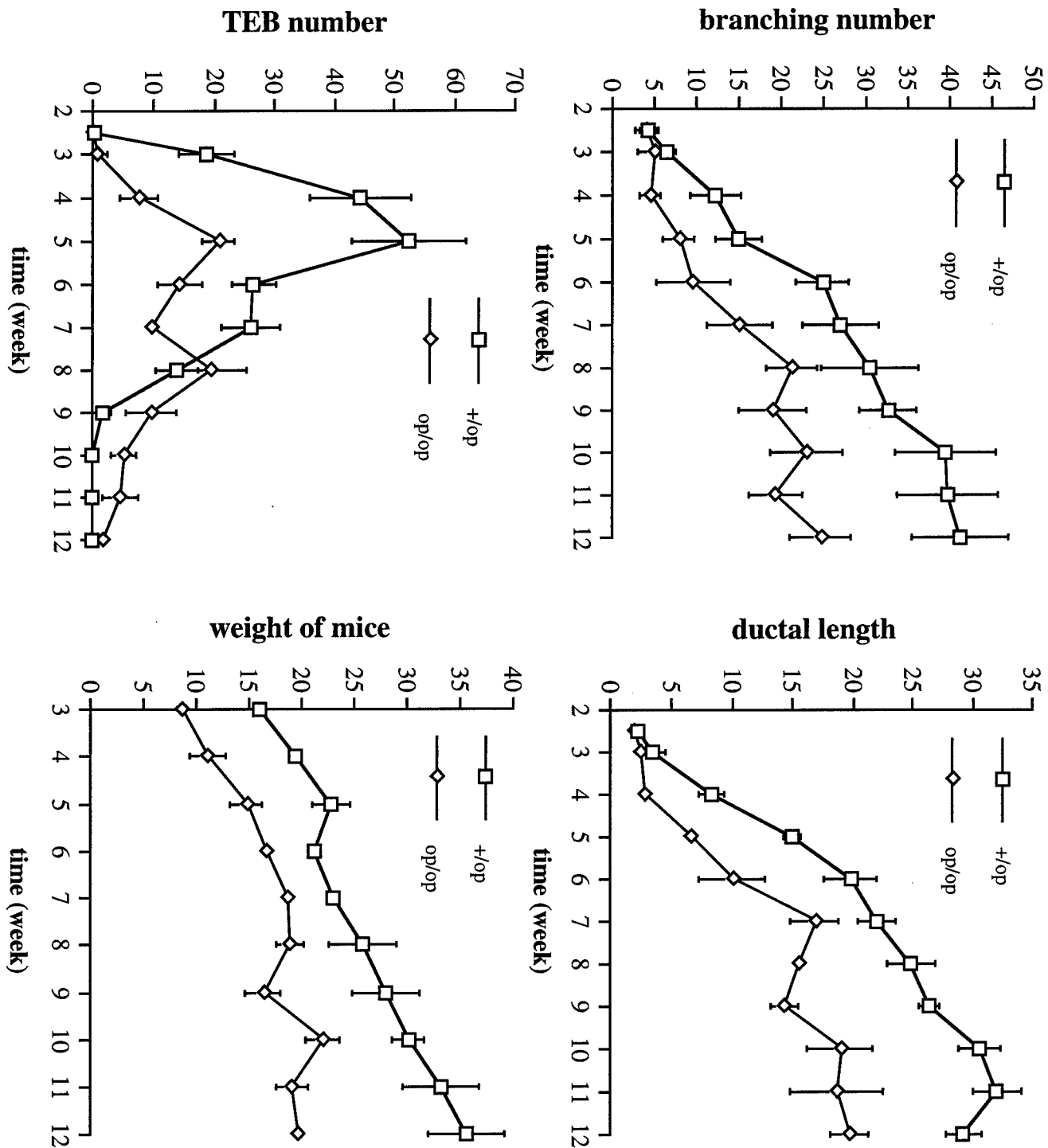


Figure 6

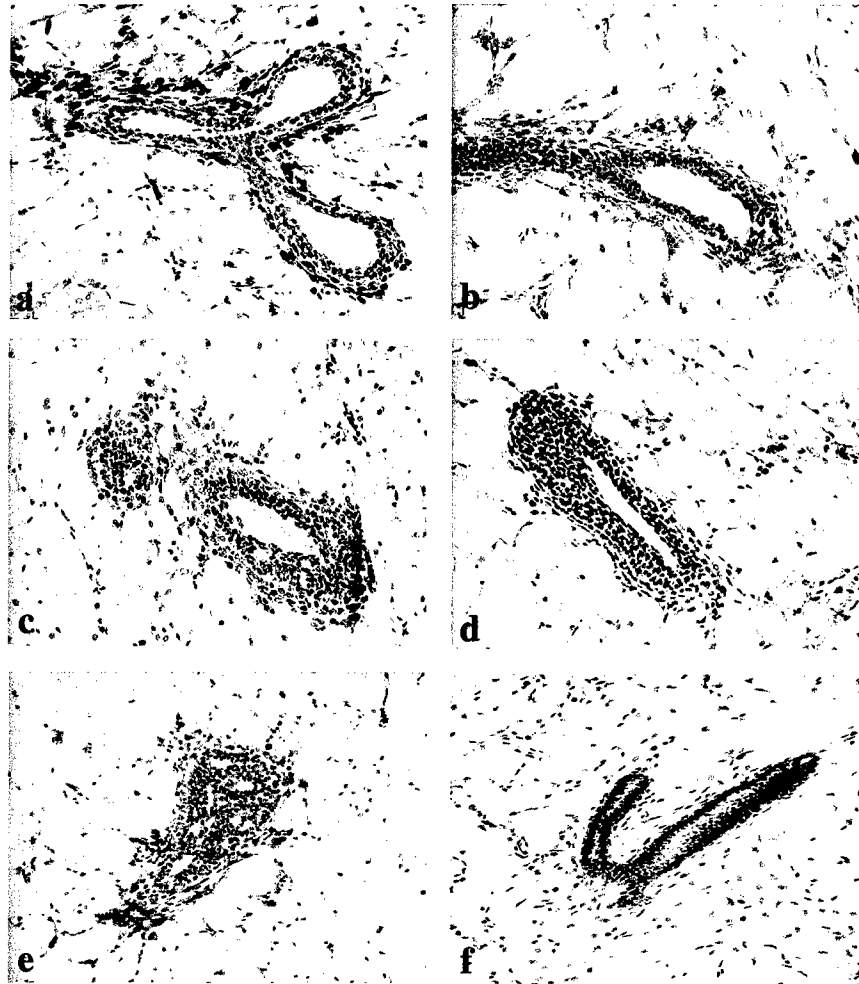
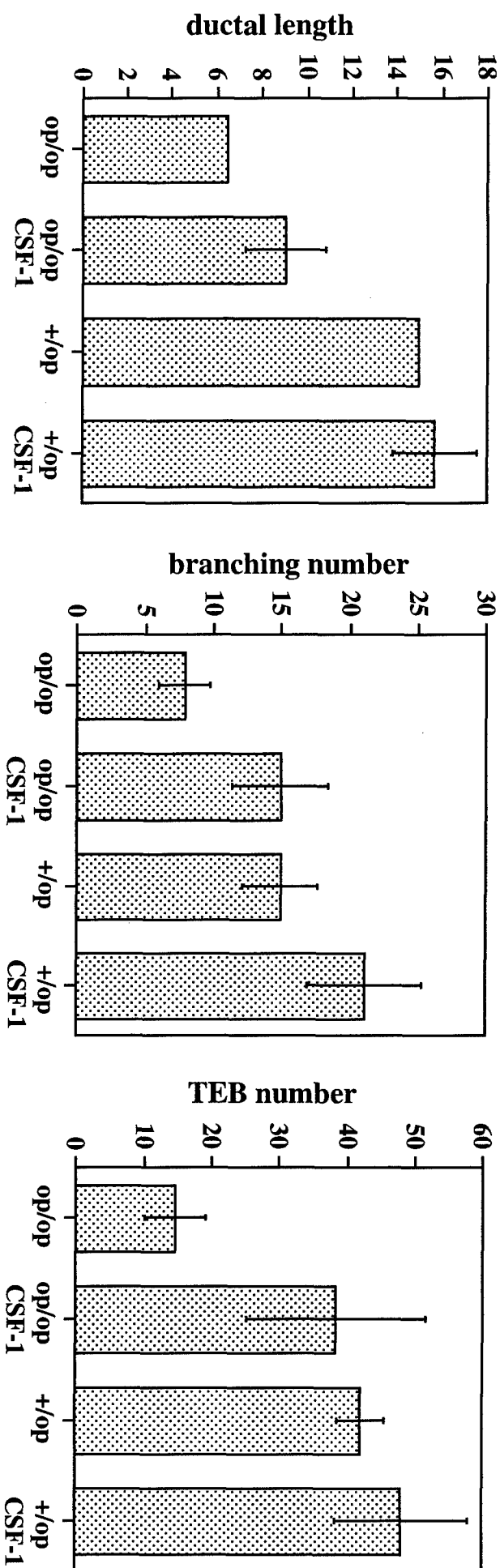


figure 7





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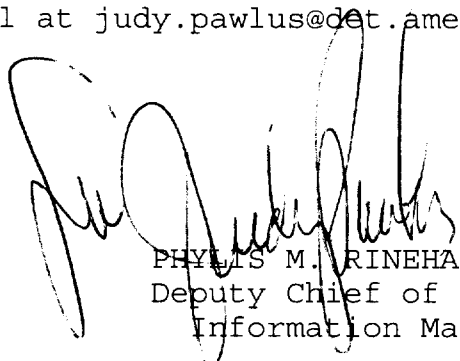
SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl



PHYLLIS M. RINEHART  
Deputy Chief of Staff for  
Information Management

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